Separation of the Osmotically Driven Fusion Event from Vesicle-Planar Membrane Attachment in a Model System for Exocytosis

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ABSTRACT We demonstrate that there are two experimentally distinguishable steps in the fusion of phospholipid vesicles with planar bilayer membranes. In the first step, the vesicles form a stable, tightly bound pre-fusion state with the planar membrane; divalent cations (Ca^{++}) are required for the formation of this state if the vesicular and/or planar membrane contain negatively charged lipids. In the second step, the actual fusion of vesicular and planar membranes occurs. The driving force for this step is the osmotic swelling of vesicles attached (in the pre-fusion state) to the planar membrane. We suggest that osmotic swelling of vesicles may also be crucial for biological fusion and exocytosis.

In the preceeding paper (8) we described the conditions for obtaining fusion of phospholipid vesicles with planar bilayer membranes. We proposed that there are two steps in the fusion process: first, the attachment of vesicles to the planar membrane; if the vesicular or planar membranes contain negatively charged lipids, this step is mediated by divalent cations (e.g., Ca^{++}). Second, the osmotic swelling of these attached vesicles, with subsequent rupture of vesicular and planar membranes in the region of contact leading to fusion. This step is driven by an osmotic gradient across the planar membrane, with the vesicle-containing *cis* side hyperosmotic with respect to the opposite *trans* side.

In this paper we show that the attachment step can be experimentally separated from the osmotically induced fusion step, and we relate the parameters affecting fusion to one or the other of these two steps. We also directly demonstrate that it is osmotic swelling of attached vesicles, and not water flow *per se* across the planar membrane, that leads to fusion. In the Discussion and Appendices we propose a theory for fusion in this model system that attempts to deal with the region of contact and the forces operating there.

MATERIALS AND METHODS

The procedures and materials for making planar membranes and vesicles, and the technique for monitoring fusion, are as described previously (8). For those experiments requiring stachyose to be trapped within the vesicles, the following method for making multilamellar vesicles was employed: 10.05 mg of a porin-lipid mixture (8 mg egg PC, 2 mg bovine PS, 50 μg porin) was suspended in hexane in a round bottom flask. The mixture was dried by rotary evaporation at room temperature and then shaken for a few minutes with three glass beads in 1 ml of a solution containing 200 mM stachyose, 100 mM KCl, 2 mM $MgCl_2$, 10 mM MES, 0.1 mM EDTA, pH 6.0. The vesicles were allowed to swell for about an hour and were then extruded through a 0.2-μm nucleopore filter (22).

RESULTS

Pre-fusion State

EXISTENCE OF THE STATE: Fig. 1A presents a clear demonstration that the step involving attachment of vesicles to the planar membrane is separable from the actual fusion event. Vesicles (phosphatidylcholine/phosphatidylethanolamine [4:1]),[1] followed by Ca^{++}, were added to the *cis* side of an asolectin "hydrocarbon-free" membrane. They were subsequently removed a few minutes later by perfusion of the *cis* compartment with Ca^{++}-containing buffer. When an osmotic gradient was then imposed across the membrane, a burst of fusion lasting <1 min occurred (rather than the continuous fusion shown earlier [8]), thus demonstrating the presence of a population of vesicles which remained attached to the planar membrane after perfusion and were capable of fusing with it. We shall describe such vesicles as being in a "pre-fusion" state.

ROLE OF CALCIUM: With negatively charged lipids, cal-

[1] *Abbreviations used in this paper:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; and PS, phosphatidylserine.

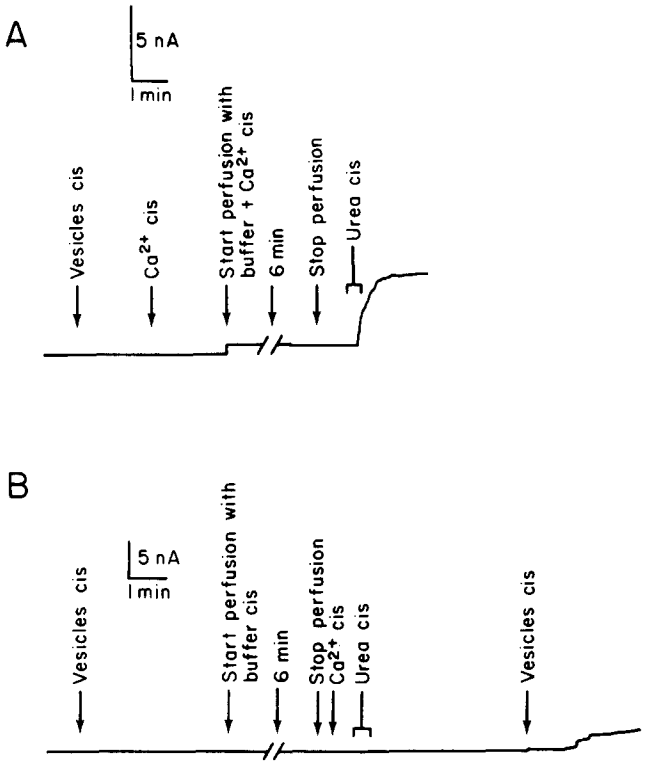

FIGURE 1 Demonstration of the existence of the pre-fusion state. (*A*) An asolectin "hydrocarbon-free" membrane is clamped at V = 20 mV and separates symmetric solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). At the start of the record, vesicles (PC:PS, 4:1; ~10^{11}/ml) are added to the *cis* compartment. 2 min later, $CaCl_2$ is added to the *cis* compartment to a concentration of 15 mM. Perfusion of the *cis* compartment with the original solution +15 mM $CaCl_2$ is begun 2 min later and continued for 8 min, during which 7 vol (15 ml) of the compartment are exchanged. (The shift in the baseline at the start of perfusion is an artifact.) 1 min after perfusion is stopped, an osmotic gradient is formed by the addition of urea to the *cis* side to a concentration of 450 mM. A brief burst of fusion occurs (<1 min), thus demonstrating a population of vesicles, capable of fusion, that remained attached to the membrane after perfusion of free vesicles out of the *cis* compartment. A few fusion events occur after the end of the burst. (*B*) Demonstration of the necessity of Ca^{++} for the formation of the prefusion state. An asolectin "hydrocarbon-free" membrane clamped at V = 20 mV separates symmetrical solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). At the start of the record, vesicles (PC:PS, 4:1; ~10^{11}/ml) are added to the *cis* side, and 4 min later perfusion of that side is begun. 7 vol (14 ml) of solution are perfused through the *cis* compartment during the subsequent 8 min. Thus, the conditions are virtually identical to those in *A*, except Ca^{++} was not added to the *cis* side before or during perfusion of the *cis* compartment. After perfusion is stopped, $CaCl_2$ (15 mM) is added to the *cis* side. When urea is now added to the *cis* side to a concentration of 450 mM, no fusion occurs, in contrast to the result in *A*, indicating that there are no vesicles in the pre-fusion state. When fresh vesicles are added to the *cis* side, fusion begins, thus demonstrating (as a control) that these vesicles are capable of fusing with this membrane.

cium is required to form this pre-fusion state. If the experiment in Fig. 1*A* is repeated without addition of calcium to the vesicle-containing *cis* compartment, no burst of fusion occurs when the osmotic gradient is subsequently applied after perfusion (Fig. 1*B*). Once the pre-fusion state is formed, however, the continued presence of Ca^{++} in the aqueous phase is not required for its maintenance; some of the vesicles in this state remain stable even in a calcium-free 1 mM EDTA medium (Fig. 2).

Although divalent cations are required to obtain high rates of fusion between negatively charged vesicles and membranes, the osmotic gradient alone suffices if both are uncharged (8). If a similar pre-fusion state also occurs in this instance, it should do so in the absence of calcium; this expectation is realized (Fig. 3).

In summary, under those conditions in which fusion occurs in the presence of an osmotic gradient, the vesicles can still form a tightly bound state with the planar membrane in the absence of the gradient. (In our earlier work we could not document such a state [9], probably because we were dealing with so few a number of fusion events that we usually could not detect it.) This state of tight, vesicle-membrane binding induces no measureable change in the electrical conductance of the planar membrane; the integrity of the planar membrane is apparently preserved, whatever the exact nature of the vesicles' interaction with it. In addition, there must be a deep energy minimum associated with this state, as it is stable over many minutes.

HETEROGENEITY OF VESICLES IN THE PRE-FUSION STATE: There appear to exist different populations of vesicles in the pre-fusion state capable of fusing with the planar membrane. This is illustrated in Fig. 2, where successive increases in the osmotic gradient result in successive bursts of fusion; i.e., increasing the osmotic gradient recruits larger numbers of vesicles into the "fusable" population. These results complement experiments on the influence of the magnitude of the osmotic gradient on fusion rate (see below, *Magnitude of Osmotic Gradient and the Rate of Fusion*). They also may explain why the rate of fusion decreases with time and fusion eventually ceases (8, 9). Apparently, vesicles can bind to the planar membrane but, for various reasons (perhaps related to their size), do not fuse. Such vesicles obstruct the membrane surface, preventing new vesicles from reaching it. As free surface area decreases, fusion rate declines, until ultimately no accessible membrane remains, and fusion ceases. This interpretation is supported experimentally. If the vesicles in solution are removed via perfusion after fusion has ceased (maintaining ionic conditions and gradients) and new vesicles are now added, no new fusion is observed. (Control experiments show that planar membranes bathed in $CaCl_2$ and osmoticants for 90 min without vesicles will still support fusion when vesicles are added. Planar membranes bathed with vesicles and $CaCl_2$ for 2 h will still support fusion when osmoticants are added, in a single burst which quickly ceases.) We conclude that cessation of fusion is caused by vesicles that have adsorbed to but not fused with the planar membrane, thereby occluding the planar membrane so that "fusible" vesicles cannot approach.

Effect of Stirring on Fusion with Decane-containing Membranes

For fusion to occur with decane-containing membranes, the *cis* compartment must be stirred (8). This effect of stirring relates to the pre-fusion state and not to the actual fusion event associated with osmotic entry of water into the vesicles (Fig. 4). With vesicles and Ca^{++} present in the *cis* compartment, stirring in the absence of an osmotic gradient does not cause fusion; when stirring is now stopped, the establishment of an osmotic gradient (by replacing the *trans* compartment with a hypoosmotic medium) results in a burst of fusion. The

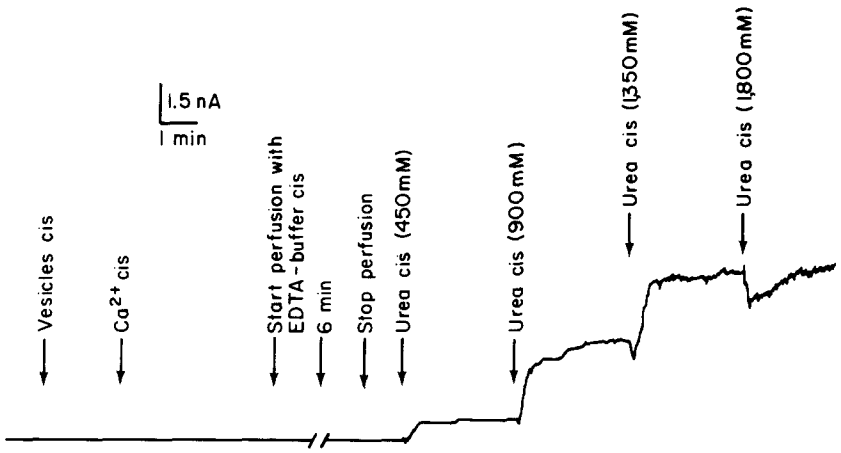

FIGURE 2 Demonstration that, once formed, the pre-fusion state is stable in the presence of calcium-free, 1 mM EDTA. An asolectin "hydrocarbon-free" membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). At the start of the record, vesicles (PC:PS, 4:1; ~10^{11}/ml) are added to the *cis* side, and 2 min later, $CaCl_2$ (15 mM) is added. 4 min later, perfusion of the *cis* side is begun with EDTA buffer (100 mM KCl, 10 mM MES, 1 mM EDTA, pH 6.0). Perfusion continues for 8 min, during which time 7 vol (15 ml) of EDTA buffer are perfused through the compartment. 1 min after perfusion is terminated, an osmotic gradient is formed by the addition of urea to the *cis* side to a concentration of 450 mM. A burst of fusion occurs, indicating that vesicles have remained in the prefusion state in the presence of calcium-free, 1 mM EDTA. At 3-min intervals, urea is added to the *cis* side, raising the concentration to 900, 1,350, and 1,800 mM. A burst of fusion follows each of these additions, though it is much smaller after the osmotic gradient is increased to 1,800 mM. (The sudden decreases in conductance following the last two urea additions are probably caused by dilution of salt in the *cis* compartment by the large volumes required for these additions.)

cessation of fusion after the burst presumably results from depletion of fusable vesicles in contact with the planar membrane, as further stirring of the *cis* side results in further fusion. If the *trans* side is now made isosmotic but the *cis* side not stirred, re-establishing an osmotic gradient by again perfusing the *trans* side with a hypoosmotic medium does not result in fusion. Presumably, fusable vesicles are not in contact with the membrane. Stirring of the *cis* side, however, results in the resumption of fusion. Our current hypothesis is that the role of stirring is to sweep vesicles in from the toroidal region where possibly the actual attachment of vesicles to the planar membrane occurs in decane-containing membranes.

Magnitude of Osmotic Gradient and the Rate of Fusion

The rate of fusion is a sensitive function of the magnitude of the osmotic gradient. This is illustrated in Fig. 5 and in Table I. In these experiments, an initial fusion rate was obtained with a 500 mosM gradient, and the subsequent decline in rate was recorded as the gradient was reduced by addition of osmoticant to the *trans* compartment. Halving the osmotic gradient from 500 to 250 mosM reduces the rate of fusion by almost an order of magnitude (Table I). The smallest gradient that induces measurable fusion rates is ~100 to 150 mosM for decane-containing membranes and ~200 mosM for "hydrocarbon-free" membranes. This result may reflect differences in the water permeability of the two types of membranes.

Similar data were obtained whether urea, glycerol, glucose, or KCl were used as osmoticants. In contrast, essentially no fusion was induced by formamide or ethyleneglycol gradients even as large as 750 mosM. Nor did these solutes inhibit fusion (induced by other osmoticants) when added to the *trans* side. The ineffectiveness of these solutes is a consequence of their relatively high membrane permeability; a theoretical justification for this statement is reserved for a future communication.

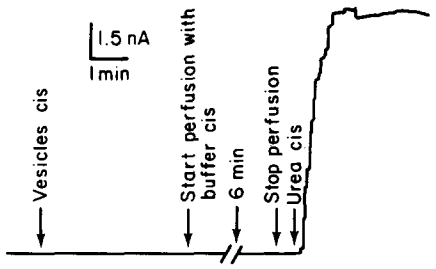

FIGURE 3 Demonstration that the prefusion state forms between uncharged vesicles and an uncharged membrane in the absence of calcium. An *E. coli* PE "hydrocarbon-free" membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). At the start of the record, vesicles (egg PC:egg PE, 1:1; ~10^{11}/ml) are added to the *cis* compartment. 4 min later, perfusion of this compartment is begun. Perfusion continues for 8 min, during which time 7 vol (14 ml) are perfused through the *cis* compartment. The perfusion is stopped, and a 450 mM osmotic gradient is formed by addition of urea to the *cis* side. A burst of fusion follows, indicating that uncharged vesicles have formed a prefusion state with an uncharged membrane in the absence of calcium.

Osmotic Swelling of Vesicles Is Required for Fusion

We have continually emphasized the importance of osmotic gradients across the planar membrane in vesicle-planar membrane fusion, and have tacitly assumed that the fusion mechanism involves osmotic entry of water into the vesicles (8, 9). If this assumption is correct, any procedure that induces osmotic swelling of vesicles attached to the planar membrane should lead to fusion, even if no osmotic gradient is created

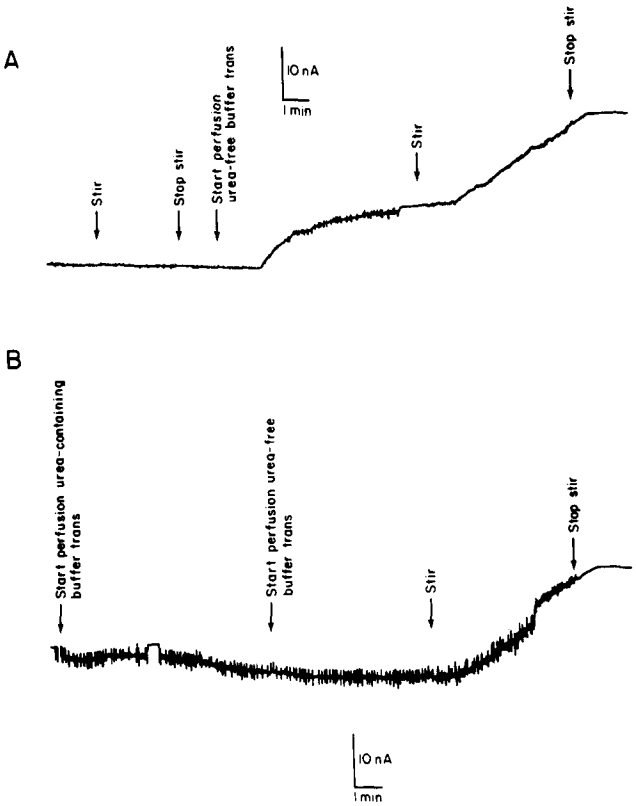

FIGURE 4 Demonstration that stirring affects the formation of the pre-fusion state, but not the actual fusion step, in decane-containing membranes. A decane-containing membrane (diphytanoyl PC: cerebroside, 7:1) clamped at V = 20 mV separates symmetric solutions (200 mM urea, 100 mM KCl, 10 mM MES, 1 mM EDTA, pH 6.0). 15 min before the start of the records, vesicles (PC:PS, 4:1; ~10^{10}/ml) and 10 mM $CaCl_2$ were added to the *cis* compartment. (*A*) In the absence of an osmotic gradient, both compartments are stirred for 3 min. 1½ min after stirring has ceased, urea-free buffer (100 mM KCl, 10 mM MES, 1 mM EDTA, pH 6.0) is perfused through the *trans* compartment, forming an osmotic gradient. A burst of fusion occurs. Following this, in the presence of the osmotic gradient, stirring is begun. About 1½ min later, fusion begins and continues at a steady rate until the cessation of stirring 6 min later, at which time fusion stops. (*B*) Urea-containing buffer is now perfused into the *trans* compartment, abolishing the osmotic gradient. This time the compartments are not stirred in the absence of an osmotic gradient. After 8 min, urea-free buffer is perfused into the *trans* compartment, forming again an osmotic gradient. No fusion occurs during the next 7 min until stirring is resumed. At this time fusion resumes, indicating that the lack of fusion was not due to having reached the plateau. Part *B* of this experiment indicates that the burst of fusion seen in part *A* was not caused by the perfusion process, but was related to the stirring that preceded the formation of the osmotic gradient.

across the planar membrane. This expectation has been realized (Fig. 6 and reference 7).

Multilamellar vesicles loaded with stachyose were added to the *cis* compartment containing the vesicle-loading solution; the *trans* compartment contained an isosmotic solution. Thus, the solutions in the *cis* and *trans* compartments and within the vesicles were of identical osmolality. After the prefusion state had been formed, stachyose was replaced in the *cis* compartment by isosmotic urea; no osmotic gradient was thereby created and no net water flow occurred across the planar bilayer. However, since the vesicle membranes were permeable to urea but not to stachyose (both because of their

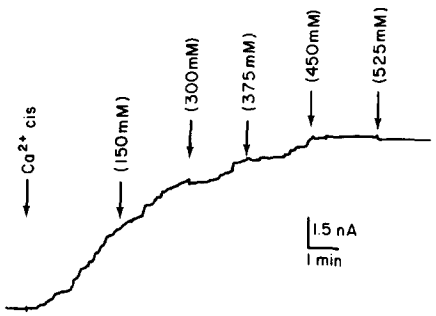

FIGURE 5 Dependence of the fusion rate on the magnitude of the osmotic gradient. An asolectin "hydrocarbon-free" membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). Before the start of the record, vesicles (PC:PS, 4:1; ~10^{11}/ml) were added to the *cis* compartment, followed by 600 mM urea. At the start of the record, $CaCl_2$ is added to the *cis* side to a concentration of 15 mM, and fusion begins. 3 min later, urea is added to the *trans* side to a concentration of 150 mM, and in succeeding 2-min intervals it is again added to the *trans* side, raising the concentration successively to 300, 375, 450, and 525 mM. Each of these urea additions decreases the osmotic gradient, and with each reduction of the osmotic gradient, the fusion rate decreases.

TABLE I

Dependence of the Fusion Rate on the Magnitude of the Osmotic Gradient

Osmotic gradient	Average % of rate with 500 mosM gradient ± S.E.	No. of experiments
mosM		
400	55% ± 5.8	8
300	28 ± 3.6	16
250	14 ± 2.5	10
200	7 ± 1.2	5

intrinsic urea permeability and because of the porin channels [21]), urea entered the vesicles followed by water. Consequently, the vesicles swelled, and fusion resulted. (Because of the relatively slow entry of urea and the multilamellar nature of the vesicles, swelling [and subsequent fusion] occurred over several minutes, rather than in a short burst.) This experiment demonstrates that it is osmotic swelling of the vesicles, not water flow across the planar membrane, that causes fusion.

In numerous experiments with vesicles made in ≥200 mM stachyose or raffinose we always obtained fusion, as in Fig. 6. In three experiments with vesicles made in only 100 mM stachyose, we obtained no fusion. This suggests that a minimum osmotic pressure is required for vesicle-planar membrane fusion. The implications of this are considered in the Discussion.

DISCUSSION

The major finding reported here is that there are two experimentally distinguishable steps in the fusion of phospholipid vesicles with planar lipid bilayer membranes. In the first step, the vesicle approaches the planar membrane and, under certain conditions, forms with it a stable, tightly bound prefusion state. In the second step, osmotic swelling of the vesicle in the prefusion state leads to destabilization of the vesicular and planar membranes in the region of contact and to their subsequent fusion. We consider each of these steps in turn.

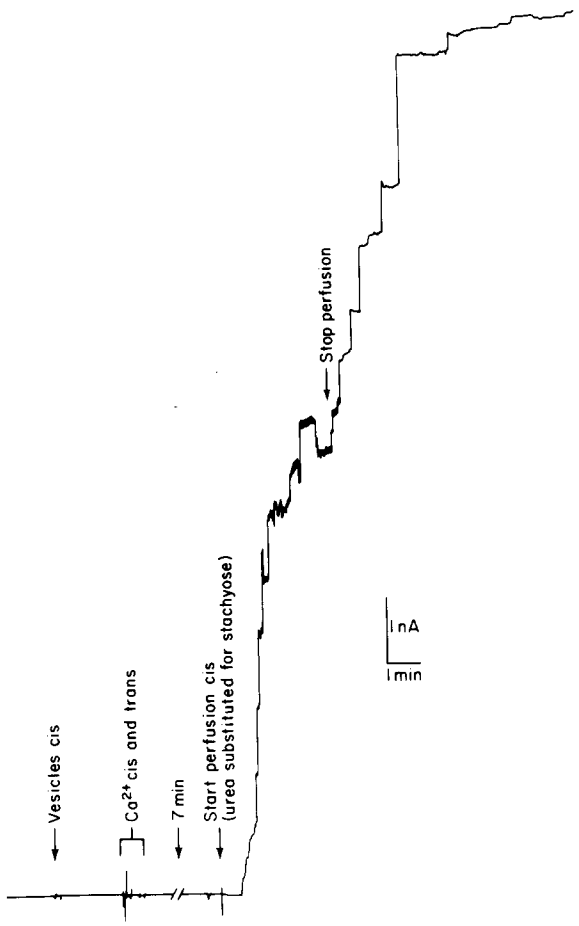

FIGURE 6 Demonstration that vesicular swelling alone, without an osmotic gradient across the planar membrane, will lead to fusion. An asolectin "hydrocarbon-free" membrane clamped at V = 20 mV separates isosmotic solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0 in both compartments; in addition, the *cis* solution contains 200 mM stachyose and the *trans* solution contains 200 mM glucose). At the start of the record, multilamellar vesicles (PC:PS, 4:1, ~10^{10}/ml) loaded with a solution of the same composition as that of the *cis* solution are added to the *cis* compartment. 2 min later $CaCl_2$ is added to the *cis* and *trans* compartments to a concentration of 15 mM. After 9 min (during which time a population of vesicles has had time to enter the prefusion state), the *cis* compartment is perfused with a vesicle-free solution of the same composition as that already present in the compartment, except that 200 mM urea replaces the 200 mM stachyose. After ~45 s, a burst of fusion begins that lasts for several minutes. Note that fusion occurs in the absence of an osmotic gradient across the planar membrane (see text for explanation).

The Pre-fusion State

STABILITY: Our ability to identify the pre-fusion state resulted from its rather unexpected stability: once formed, it persists for at least 15 min, our longest observation. (Although we did not explicitly attempt to determine the duration of this state, the following observation indicates that it is stable for longer periods of time. After the fusion rate in the presence of vesicles and an osmotic gradient has decreased with time and fusion has finally ceased, if the vesicles in solution are removed and replaced with freshly added vesicles to the *cis* side, the planar membrane remains occluded, and fusion does not resume even after 45 min.) Ca^{++} (divalent cation) is required for the *formation* of the pre-fusion state between negatively charged membranes, but not for its *maintenance*; even a Ca^{++}-free 1 mM EDTA solution does not disrupt the association of all the vesicles with the planar membrane.

CLOSENESS OF VESICULAR AND PLANAR MEMBRANES: The fact that an osmotic gradient across the planar bilayer can drive fusion indicates that the vesicle and planar membrane are in close contact in the pre-fusion state. Even if they were only 5 Å apart, 80% of the water flowing across the planar membrane in the region of its association with the vesicle membrane would be shunted through the aqueous space between the two membranes and would not enter the vesicle to induce swelling and subsequent fusion (Appendix I). Measurements of lamellae spacing in PC and PE mutlilayers show that the equilibrium separation distances are 27 and 20 Å, respectively (18, 30). These equilibrium distances result from the balance between attractive van der Waals forces and the repulsive "hydration forces" that arise from the need to remove water from between the two membranes when they approach (26). Our observation that the fusion rates of PC vesicles with "hydrocarbon-free" PC membranes are much lower than those of PC:PE vesicles with "hydrocarbon-free" PE membranes (8) is consistent with the ethanolamine group being less strongly hydrated than the choline moiety (34) and with the consequent closer spacing of PE multilayers (20 Å) than of PC multilayers (27 Å).

There is an enormous energy barrier, interpreted as arising from hydration forces, that prevents bilayers from approaching significantly closer than their equilibrium distance (18). For negatively charged membranes, however, divalent cations (particularly Ca^{++}) can decrease this energy. Not only does Ca^{++} reduce electrostatic repulsion between negatively charged bilayers (this effect only reduces bilayer repulsion to the high levels seen with neutral lipids), it also binds to the charged head groups, thereby dehydrating them and thus allowing the bilayers to come into close contact (29). This effect can contribute to Ca^{++}-induced fusion between negatively charged vesicles and planar membranes.

A possibility that we cannot preclude is that the close contact between vesicle and planar membrane occurs in the border region, where bilayer merges with monolayer in "hydrocarbon-free" membranes and with bulk torus in decane-containing membranes. The requirement of stirring in the *cis* compartment for formation of the pre-fusion state with decane-containing membranes suggests this possibility. In the border region with its sharp changes in curvature and the presence of monolayer, the hydration energy barrier may be sufficiently low that intimate vesicle-membrane contact can occur there.

RELATIONSHIP OF PRE-FUSION STATE TO VESICLE-VESICLE AGGREGATION: Although the pre-fusion state involves aggregation between two membranes, its relationship to the much studied vesicle-vesicle aggregation is somewhat problematic. In particular, whereas vesicles made from PC:PS (4:1) readily fuse in 10 mM Ca^{++} with "hydrocarbon-free" bilayers of the same composition, they do not even aggregate (let alone fuse) with each other in 10 mM Ca^{++} (11; unpublished observations). Similarly, uncharged PE:PC vesicles do not aggregate with each other, but do form the pre-fusion state and fuse with PE:PC planar membranes. What accounts for these differences?

The possible role of the border region in vesicle-planar membrane association has been discussed earlier. Additionally, if the planar membrane is somewhat flexible and disten-

sible, the vesicle could invaginate into it and thereby increase the area of contact between the two membranes (Fig. 8 in Appendix II). Even if not dehydrated, the larger the area of contact, the greater the decrease in energy and the more stable the interaction. There is no comparable process by which two spherical vesicles can increase their area of contact and thus stabilize their interaction. In other words, membranes will associate only if the decrease in energy per unit area of contact times the area of contact is sufficient to overcome the disordering effect of thermal energy (kT). Because of the large area of contact, vesicle-planar membrane interaction is stabilized (see Appendix II), whereas because of the small region of contact between two spheres, vesicle-vesicle interaction is not.

Evans and Kwok (12) performed an experiment that illustrates the above idea. Two vesicles made of PC:PS (10:1) were held in suction pipettes and pushed together in the presence of 10 mM Ca^{++}. They did not form a stable interaction and were easily separated. If, however, the vesicles were first shrunk osmotically so that they became "flacid" and were then pushed together in 10 mM Ca^{++}, they tightly adhered to each other. This experiment demonstrates that the area of interaction is an important parameter of the stability and strength of that interaction. Thus, some of the differences between the results obtained in vesicle-vesicle and vesicle-planar membrane experiments may reflect differences in the geometry of interaction.

The Fusion Step

The actual fusion of vesicle and planar membrane, as opposed to their attachment (the prefusion state), is driven by osmotic swelling of the vesicle. Since generally this was achieved by application of an osmotic gradient across the planar membrane, there was the possibility that water flow per se across that membrane played a role in the process. In this paper, however, we have directly demonstrated, in the absence of an osmotic gradient across the planar membrane, that it is osmotic swelling of attached vesicles that causes fusion (see also reference 7).

When swelling of vesicles is induced by imposing an osmotic gradient across the planar membrane, the driving forces for water flow are as follows. The osmotic gradient across the planar membrane induces water flow from the *trans* side into the *cis* side, including the aqueous corridor between the vesicles in the pre-fusion state and the planar membrane. As discussed in Appendix I, no concentration gradient of solute develops between the corridor and the *trans* side and/or the vesicle interior. In other words, if there is a corridor, water is not driven into a vesicle via osmosis. As water flows into the restricted space (the corridor) between vesicle and planar membrane, a pressure develops within the corridor, because water is incompressible. The vesicle and planar membrane are not significantly pushed apart, because of the restoring van der Waals interaction. Thus, if there is a corridor, it is the pressure that develops there, rather than concentration gradients, which force water both around and into the vesicle. If there is not a corridor, osmosis of water across the combined planar-vesicle membrane results in swelling of vesicles.

As a result of the flow of water into the vesicles, independent of how it is accomplished (be it from osmotic gradients across the planar membrane or from the stachyose experiments), the vesicles swell. Once a vesicle is fully swollen, changes in volume as a result of flow of incompressible water into the vesicle are minimal, but a pressure resisting further flow of material into the vesicle develops (see below). Our picture is that there exists a critical pressure for a given vesicle, below which fusion does not occur. Therefore, the hydrostatic pressure that develops within the swollen vesicle is the driving force for fusion.

The details of this final fusion step remain to be elucidated. We envision that osmotic entry of water into a vesicle causes stretching of the vesicular and planar membrane in their region of tight contact. The subsequent exposure of hydrophobic domains then leads to melding of the two apposed bilayers (fusion) and discharge of vesicular contents across the planar membrane. Because of the sharp curvatures and the possible dehydrated state in the region of contact, vesicle rupture may preferentially occur there. There is no evidence, however, on this point, and many attached vesicles may rupture without fusing to the planar membrane, discharging their contents back into the *cis* compartment. The present assay does not score such events.

It is noteworthy that vesicles can sustain a certain osmotic stress without fusing to the planar membrane. In Fig. 2 we saw that although an osmotic gradient of 450 mosM led to a burst of fusion, there still remained a population of vesicles in the pre-fusion state that survived the stress, as evidenced by further bursts of fusion when the osmotic gradient was increased. This must mean that the vesicles can support a hydrostatic pressure difference across their membranes. (Direct experimental support for this statement comes from the observation that when vesicles made in a 1,000 mosM solution are placed in a 500 mosM solution, less than 50% of them lyse, suggesting that many of the vesicles can support a hydrostatic pressure difference of more than 10 atmospheres (2). If the vesicles were not spherical initially, however, their failure to lyse in the hypoosmotic medium is at least partially attributable to their swelling to spherical shape, and not to their ability to support a 500 mosM pressure difference across their membranes.) Otherwise, all of the vesicles would have continued to swell and eventually burst under the initial osmotic gradient, since they are permeable to the solute used to impose the gradient (because of the porin channels in their membranes).

This ability of the vesicle to sustain a hydrostatic pressure difference across its membrane is directly demonstrated in the stachyose-urea experiments (see Fig. 6). It is clear there that in the absence of a counteracting hydrostatic pressure difference, water and urea cannot achieve equilibrium across the vesicle membrane, and therefore the vesicle will swell indefinitely until it bursts. In fact, however, with only 100 mosM (instead of 200 mosM) stachyose trapped inside the vesicles, fusion did not take place. A counteracting hydrostatic pressure difference must have built up to oppose osmotic water entry. (The magnitude of this hydrostatic pressure difference is not necessarily 2.24 atm, the osmotic pressure corresponding to 100 mosM. Because stachyose may adsorb to lipid bilayers, the concentration of "free" stachyose in the interlamellar spaces of the multilamellar vesicles used in these experiments may be significantly lower than 100 mosM.) The hydrostatic pressure difference that a vesicle can sustain across its membrane is related both to its size (the smaller the vesicle the larger the pressure) and to the elastic forces that develop after the vesicle has reached a spherical state (15).

Relevance to Biological Fusion

In considering the possible implications of our results to biological systems, we must distinguish between the two steps in the fusion process: (*a*) the formation of the pre-fusion state

and (*b*) the osmotic stress on the vesicles that results in actual fusion.

(*a*) It is clear that at least some portion of the two biological membranes must contact each other before coalescing, but whether a stable pre-fusion intermediate exists, its lifetime, size, and similarity to the pre-fusion state described above is uncertain. Initial electron microscopic examination of chemically fixed thin sections of many fusing systems revealed large areas of a pentalaminar structure ~130 Å in thickness (14, 16, 17, 24, 32). Subsequent examination of mast cells and sea urchin eggs by quick-freezing and freeze-fracture suggested that the area and lifetime of this putative intermediate is artifactually increased by chemical fixation (5, 6). Nevertheless, in quick-frozen, freeze-substituted thin sections of *Limulus* amoebocytes, small areas of this pentalaminar structure are seen soon after stimulation, and pore formation appears to occur in such areas (23). This junction is consistent with the prefusion state described above. Our ability to separate adherence and swelling allowed us to stabilize the prefusion state. Ultrastructural examination of hypertonically inhibited fusion systems may yield images of such an intermediate.

(*b*) The role of osmotic forces in biological fusion events deserves further consideration. Pollard and his colleagues (3, 27, 28) originally proposed this as the driving force for fusion of chromaffin granules and other intracellular granules to plasma membranes. There are several examples of vesicle swelling associated with exocytosis (4, 23, 31; and W. W. Douglas, quoted in reference 28), but a causative link between swelling and fusion has yet to be demonstrated. Recently it was shown that osmotic gradients can modulate vasopressin-induced fusion in toad urinary bladder in a manner consistent with the results in our model system (13). Similar experiments should be attempted in other preparations.

The trigger for osmotic water entry into exocytotic vesicles is at present unknown, but a strong candidate is Ca^{++} (which may act both in this process and in the formation of the pre-fusion state). Our urea-stachyose experiment illustrates how Ca^{++} might function in this capacity. In that experiment an impermeant solute, stachyose, was replaced in the medium by a permeant one, urea. The entry of the latter into the vesicle then led to osmotic swelling. Analogously, a calcium-induced permeability change of the vesicle membrane to ions could lead to osmotic swelling of cytoplasmic vesicles in the pre-fusion state. The ubiquitous calcium-modulated potassium, and nonselective cation, channels (1, 10, 19, 20, 25, 35) offer one obvious possible way for this to happen. Alternatively, calcium could solubilize osmotically inactive material within the vesicle, or activate an enzyme that could generate osmotically active particles within the vesicle from the osmotically inactive material there, and thereby lead to vesicle swelling; experiments with pulmonary mast cells suggest such a mechanism (4).

APPENDIX I

Separation Distance of Vesicular and Planar Membranes in the Prefusion State

The fact that an osmotic gradient across the planar membrane can cause vesicles in the prefusion state to swell (and thereby fuse with the planar membrane) suggests that the distance of separation between vesicular and planar membranes is small. The argument is as follows: consider Fig. 7*A*, where, as discussed in Appendix II, the planar membrane is partially wrapped around the vesicle. There are two parallel routes for water that flows across the planar membrane (by osmosis) into the intermembrane space between the vesicular and planar membrane. The water can either cross the vesicular membrane (and thus be effective in causing vesicle swelling and fusion) or it can flow through the intermembrane space out into the bulk solution. The fraction of the water flowing across the planar membrane that crosses the vesicular membrane is dependent on the relative resistances of these two pathways. Let us estimate their magnitude.

For the sake of simplicity, we model the flow of water along the spherical corridor (depicted in Fig. 7*A*) between the planar and vesicular membranes as flow between two parallel plates of length l and width w separated by a distance d (Fig. 7*B*). We further assume that there is a pressure difference ΔP ($= P_1 - P_2$) between the ends of the plate that is equal to the osmotic pressure differences $\Delta\Pi$ ($= RT\Delta c_s$) between the *cis* and *trans* compartments, and that this pressure difference both drives water out through the corridor into the surrounding solution and also drives it across the vesicular membrane into the vesicle. (In reality, water flows across the planar membrane, under the driving force $RT\Delta c_s - \Delta P'$, into any region of the corridor, where $\Delta P'$ is a function of the location of the region. It flows out of this region both along the corridor, under the local pressure gradient, and across the vesicular membrane into the vesicle, under the pressure difference $\Delta P'$.)[2]

The rate of flow of water, J^c, between the two parallel planes of the corridor is given for laminar flow by:

$$J^c = \frac{wd^3}{12l\eta}\,\Delta P, \tag{1}$$

where η is the viscosity of water (= 0.9 cP). The rate of flow of water, J^m, across the vesicular membrane is:

$$J^m = \frac{wl\bar{V}_{H_2O}P_f\Delta P}{RT}, \tag{2}$$

where, $\bar{V}_{H_2O}$ is the partial molar volume of water (= 18 cc/mol), P_f is the filtration (or osmotic) water permeability coefficient of the vesicular membrane, and RT has its usual meaning ($\approx 2.5 \times 10^{10}$ ergs at room temperature). From Eq. 1 and 2 we obtain:

$$\frac{J^m}{J^m + J^c} = \frac{(l^2P_f/d^3)12\eta V_{H_2O}/RT}{1 + (l^2P_f/d^3)12\eta V_{H_2O}/RT} = \frac{(8.6 \times 10^{-11}\ s)l^2P_f/d^3}{1 + (8.6 \times 10^{-11}\ s)l^2P_f/d^3}. \tag{3}$$

Eq. 3 is an expression for the fraction of the total water flowing across the planar membrane into the corridor (J^m +

[2] It can easily be shown that the concentration of solute in the corridor is essentially uniform and the same as that in the *cis* compartment. This is so, because diffusion is so rapid over the relatively small length of the corridor (which is measured in angstroms) that it maintains, in the face of the bulk flow that tends to sweep solute out from this region, the concentration of the solute in the corridor at the value in the *cis* compartment. Thus, Δc_s immediately across the planar membrane bounding the corridor is the same as the difference in solute concentration between the *cis* and *trans* solutions far from the membrane. Because of the resulting osmotic flow of water into the corridor, a pressure difference ($\Delta P'$) develops at each point. (This does not significantly force apart the vesicle and planar membrane, because of the strong restoring van der Waals interaction between the two membranes.) It is this pressure, rather than osmosis, that drives water from the corridor across the vesicle membrane.

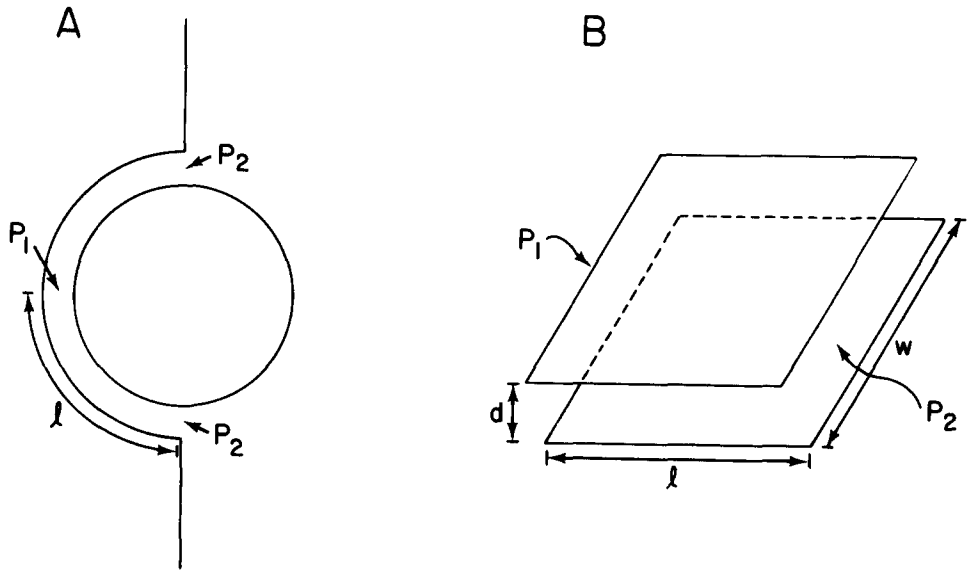

FIGURE 7

J^c) that crosses the vesicular membrane (J^m). If we consider a vesicle with a diameter of 1,000 Å and assume that in Fig. 7*A* half the vesicle surface is apposed to the planar membrane, then $l^2 \approx 1.5 \times 10^{-10}$ cm^2, and Eq. 3 becomes:

$$\frac{J^m}{J^m + J^c} = \frac{(1.3 \times 10^{-20}\ \text{cm}^2 \cdot \text{s}) P_f/d^3}{1 + (1.3 \times 10^{-20}\ \text{cm}^2\ \text{s}) P_f/d^3}. \quad (3a)$$

If $P_f = 2 \times 10^{-3}$ cm/s, a reasonable value for a lipid bilayer, then <1% of the water crossing the planar membrane crosses the vesicular membrane, if their distance apart (d) is 20 Å. At a separation distance of 5 Å, ~20% of the water crossing the planar membrane also crosses the vesicular membrane. Thus, the calculation we have gone through implies a small distance of separation of vesicular and planar membranes in the prefusion state.

APPENDIX II

Energy of Interaction between Planar and Vesicular Membrane

We shall calculate the energy of interaction between a planar membrane and a spherical vesicle; in the process we shall also determine the degree to which the former wraps around the latter, and hence the area of contact between the two membranes in the prefusion state.

Let us assume that in the prefusion state the vesicle is separated from the planar membrane by an aqueous layer of a thickness comparable to the equilibrium spacing of lamellae in multilayers—~20 Å (30). If ΔG is the van der Waals energy per unit area in bringing the vesicle from infinity to the equilibrium distance, the interaction energy is clearly increased (made more negative) by the planar membrane wrapping around the vesicle membrane and thereby increasing the area of interaction. For the wrapping around to occur, however, the area of the planar membrane must increase. (We assume that the additional phospholipids needed for the area increase some from the phospholipids dissolved in the hydrocarbon [decane, squalene, or vaseline] of the torus.) This increase of membrane area requires work to be done against the surface tension (γ) of the planar bilayer. Thus, the energy

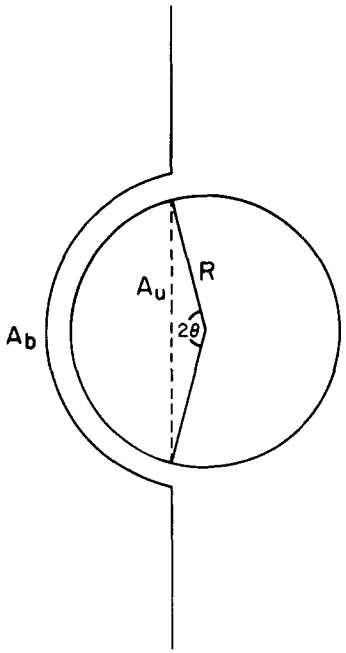

FIGURE 8

of interaction of planar and vesicular membranes is the sum of the van der Waals interaction energy (a negative quantity) and the increase in planar membrane surface energy (a positive quantity). We wish to find the degree of envelopment of the vesicle membrane by the planar membrane that minimizes this sum.

Let A_b equal the area of the planar membrane that wraps around a vesicle of radius R, A_u equal the area of the disk of planar membrane before distortion by contact with the vesicle, and 2θ equal the angle subtended from the center of the vesicle to any two diametrically opposite points on the edge of A_b (Fig. 8). The excess energy of interaction, E, resulting from the wrapping of the planar membrane around the vesicle (i.e., the energy of interaction of the configuration in Fig. 8 minus the energy of interaction of the spherical vesicle and the undistorted planar membrane) is, to a first approximation,

given by

$$E = A_b\Delta G + \gamma(A_b - A_u) \quad (1)$$

Neglecting the small distance between vesicle and planar membrane we have

$$A_u = \pi R^2 \sin^2\theta,$$

$$A_b = \int_0^\theta 2\pi R \sin \alpha R d\alpha = 2\pi R^2(1 - \cos\theta),$$

and substituting these into (1), we obtain

$$E = 2\pi R^2(1 - \cos\theta)\Delta G + \pi R^2\gamma[2(1 - \cos\theta) - \sin^2\theta]. \quad (2)$$

Setting $\frac{\partial E}{\partial \theta} = 0$, we have for the angle that minimizes the energy

$$\cos\theta = 1 + \frac{\Delta G}{\gamma}. \quad (3)$$

At an equilibrium distance of 20 Å, ΔG is between -0.01 and -0.1 erg/cm^2 (18; Parsegian, personal communication). If γ is ~2 dyne/cm (33), we obtain

$$\theta = 18^\circ, \quad \text{if} \quad \Delta G = -10^{-1} \text{ erg/cm}^2$$

$$\theta = 6^\circ, \quad \text{if} \quad \Delta G = -10^{-2} \text{ erg/cm}^2$$

If the surface tension of the planar membrane (γ) is >2 dyne/cm, the degree of contact is less (θ is smaller); if γ is <2 dyne/cm, the degree of contact is greater. Note that if $\gamma < -\Delta G$, the planar membrane will completely wrap around the vesicle. Substituting the above values of θ into Eq. 2, we obtain for a 1,000-Å vesicle:

$$E = -4 \times 10^{-13} \text{ erg} \approx -10\ kT, \qquad \theta = 18^\circ,$$

$$E = -4 \times 10^{-15} \text{ erg} \approx -0.1\ kT, \qquad \theta = 6^\circ.$$

It is clear that, depending on the values chosen for γ and ΔG, E will either be large or small compared to kT. The point that bears emphasis here is that reasonable assumptions can yield large areas of contact with energies of interaction many times kT. This will result in stable interactions between vesicles and planar membrane. In short, there is a simple and clear physical basis which can explain our experimental finding that the prefusion state is tight. In addition, if negatively charged lipids and divalent cations are present, one can expect charge binding and dehydration to further stabilize the vesicle-planar membrane interaction.

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REFERENCES

1. Adams, P. R., A. Constanti, D. A. Brown, and R. B. Clark. 1982. Intracellular Ca^{2+} activates a fast voltage-sensitive K^+ current in vertebrate sympathetic neurones. *Nature (Lond.).* 296:746–749.
2. Akabas, M. H. 1983. Fusion of unilamellar vesicles to planar lipid bilayers: a model for exocytosis. PhD Thesis Albert Einstein College of Medicine, Yeshiva University, New York.
3. Brown, E. M., C. J. Pazoles, C. E. Creutz, G. D. Aurbach, and H. B. Pollard. 1978. Role of anions in parathyroid hormone release from dispersed bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA.* 75:876–880.
4. Caulfield, J. P., R. A. Lewis, A. Hein, and K. F. Austen. 1980. Secretion in dissociated human pulmonary mast cells. Evidence for solubilization of granule contents before discharge. *J. Cell Biol.* 85:299–311.
5. Chandler, D. E., and J. Heuser. 1979. Membrane fusion during secretion. Cortical granule exocytosis in sea urchin eggs as studied by quick-freezing and freeze fracture. *J. Cell Biol.* 83:91–108.
6. Chandler, D. E., and J. Heuser. 1980. Arrest of membrane fusion events in mast cells by quick freezing. *J. Cell Biol.* 86:666–674.
7. Cohen, F. S., M. H. Akabas, and A. Finkelstein. 1982. Osmotic swelling of phospholipid vesicles causes them to fuse with a planar phospholipid bilayer membrane. *Science (Wash. DC).* 217:458–460.
8. Cohen, F. S., M. H. Akabas, J. Zimmerberg, and A. Finkelstein. 1984. Parameters affecting fusion of unilamellar phospholipid vesicles with planar bilayer membranes. *J. Cell Biol.*
9. Cohen, F. S., J. Zimmerberg, and A. Finkelstein. 1980. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes. II. Incorporation of a vesicular membrane marker into the planar membrane. *J. Gen. Physiol.* 75:251–270.
10. Colquhoun, D., E. Neher, H. Reuter, and C. F. Stevens. 1981. Inward current channels activated by intracellular Ca in cultured cardiac cells. *Nature (Lond.).* 294:752–754.
11. Duzgunes, N., S. Nir, J. Wilschut, J. Bentz, C. Newton, A. Portis, and D. Papahadjopoulos. 1981. Calcium- and magnesium-induced fusion of mixed phosphatidylserine/phosphatidylcholine vesicles: effect of ion binding. *J. Membr. Biol.* 59:115–125.
12. Evans, E., and R. Kwok. 1982. Controlled aggregation of giant PC:PS vesicles in calcium. *Biophys. J.* 37(2, Pt. 2):165*a*. (Abstr.)
13. Kachadorian, W. A., J. Muller, and A. Finkelstein. 1981. Role of osmotic forces in exocytosis: studies of ADH-induced fusion in toad urinary bladder. *J. Cell Biol.* 91:584–588.
14. Kalderon, N., and N. B. Gilula. 1979. Membrane events involved in myoblast fusion. *J. Cell Biol.* 81:411–425.
15. Kwok, R., and E. Evans. 1981. Thermoelasticity of large lecithin bilayer vesicles. *Biophys. J.* 35:637–652.
16. Lagunoff, D. 1973. Membrane fusion during mast cell secretion. *J. Cell Biol.* 57:252–259.
17. Lawson, D., M. C. Raff, B. D. Gomperts, C. Fewtrell, and N. B. Gilula. 1977. Molecular events during membrane fusion. A study of exocytosis in rat peritoneal mast cells. *J. Cell. Biol.* 72:242–259.
18. LeNeveu, D. M., R. P. Rand, and V. A. Parsegian. 1976. Measurement of forces between lecithin bilayers. *Nature (Lond.)* 259:601–603.
19. Lux, H. D., E. Neher, and A. Marty. 1981. Single channel activity associated with calcium dependent outward current in *Helix pomatis*. *Pflügers Arch.* 389:293–295.
20. Marty, A. 1981. Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature (Lond.).* 291:497–500.
21. Nikaido, H., and E. Y. Rosenberg. 1981. Effect of solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. *J. Gen. Physiol.* 77:121–135.
22. Olson, F., C. A. Hunt, F. C. Szoka, W. J. Vail, and D. Papahadjopoulos. 1979. Preparation of liposomes of defined size by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta.* 557:9–23.
23. Ornberg, R. L., and T. S. Reese. 1981. Beginning of exocytosis captured by rapid-freezing of *Limulus* amebocytes. *J. Cell Biol.* 90:40–54.
24. Palade, G. E., and R. R. Bruns. 1968. Structural modifications of plasmalemma vesicles. *J. Cell Biol.* 37:633–648.
25. Pallotta, B. S., K. L. Magleby, and J. N. Barrett. 1981. Single channel recordings of Ca^{2+}-activated K^+ currents in rat muscle cell culture. *Nature (Lond.)* 293:471–474.
26. Parsegian, V. A., N. Fuller, and R. P. Rand. 1979. Measured work of deformation and repulsion of lecithin bilayers. *Proc. Natl. Acad. Sci. USA.* 76:2750–2754.
27. Pazoles, C. J., and H. B. Pollard. 1978. Evidence for stimulation of anion transport in ATP-evoked transmitter release from isolated secretory vesicles. *J. Biol. Chem.* 253:3962–3969.
28. Pollard, H. B., K. Tack-Goldman, C. J. Pazoles, C. E. Creutz, and R. Shulman. 1977. Evidence for control of serotonin secretion from human platelets by hydroxyl ion transport and osmotic lysis. *Proc. Natl. Acad. Sci. USA.* 74:5295–5299.
29. Portis, A., C. Newton, W. Pangborn, and D. Papahadjopoulos. 1979. Studies on the mechanism of membrane fusion: evidence for an intermembrane Ca^{2+}-phospholipid complex, synergism with Mg^{2+}, and inhibition by spectrin. *Bicohemistry.* 18:780–790.
30. Rand, R. P. 1981. Interacting phospholipid bilayers: measured forces and induced structural changes. *Annu. Rev. Biophys. Bioeng.* 10:277–314.
31. Satir, B., C. Schooley, and P. Satir. 1973. Membrane fusion in a model system. Mucocyst secretion in *Tetrahymena*. *J. Cell Biol.* 56:153–176.
32. Tandler, B., and J. H. Poulsen. 1976. Fusion of the envelope of mucous droplets with the luminal plasma membrane in acinar cells of the cat submandibular gland. *J. Cell Biol.* 68:755–781.
33. Tien, H. Ti. 1974. Bilayer Lipid Membranes (BLM). Theory and Practice. Marcel Dekker, Inc. New York. 40.
34. Wilkinson, D. A., and J. F. Nagle. 1981. Dilatometry and calorimetry of saturated phosphatidylethanolamine dispersions. *Biochemistry.* 20:187–192.
35. Yellen, G. 1982. Single Ca^{2+}—activated nonselective cation channels in neuroblastoma. *Nature (Lond.).* 296:357–359.